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Article type : Review Article

Endothelial colony forming cells and proangiogenic cells: clarifying definitions and their potential role in mitigating acute kidney injury

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Short title: Endothelial dysfunction and cell therapy in AKI

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Abstract

Acute kidney injury (AKI) represents a significant clinical concern that is associated with high mortality rates and also represents a significant risk factor for the development of chronic kidney disease (CKD). This article will consider alterations in renal endothelial function in the setting of AKI that may underlie impairment in renal perfusion and how inefficient vascular repair may manifest post-AKI and contribute to the potential transition to CKD. We provide updated terminology for cells previously classified as “endothelial progenitor” that may mediate vascular repair such as pro-angiogenic cells and endothelial colony forming cells. We consider how endothelial repair may be mediated by these different cell types following vascular injury, particularly in models of AKI. We further

summarize the potential ability of these different cells to mitigate the severity of AKI, improve perfusion and maintain vascular structure in pre-clinical studies.

Key Words: Rarefaction, Angiogenesis, Ischemia, Fibrosis, Inflammation

Background

Acute kidney injury (AKI) represents a significant and growing clinical concern, which affects approximately 5% of hospitalized patients and is associated with high morbidity and mortality rates. AKI is defined as a rapid loss of renal function occurring over the course of hours or days and is primarily caused by impaired renal blood flow, nephrotoxicity or sepsis leading to ischemic or hypoxic damage ¹. In addition, AKI is associated with injury to the renal parenchyma, particularly tubular epithelial cells, but also vascular and interstitial cells. Repair mechanisms in the kidney typically mediate recovery of renal function and structure, which is observed in most surviving patients. However, it is now recognized that patients who have recovered from AKI are at increased risk for the development of either chronic kidney disease (CKD) or end-stage renal disease (ESRD) ²⁻⁴. This may result from repair processes following AKI that are either incomplete or maladaptive, predisposing the development of renal fibrosis ².

Impaired perfusion in acute kidney injury

In animal models of acute kidney injury such as ischemia-reperfusion, there is typically an impairment in renal blood flow associated with lost renal function and tissue damage ⁵. In models of sepsis, AKI may or may not occur in the presence of reduced renal blood flow, and this has been an area of some controversy ⁶. The use of contrast media is another significant risk factor predisposing AKI. Contrast media is thought to promote vasoconstriction of pericytes, leading to an exacerbation of hypoxia in the renal medulla ⁷.

Regardless of effects on total renal blood flow, it is thought that heterogeneous disturbances in microvascular flow patterns are likely evident in AKI of various etiologies⁶. Such impairment at the microvascular level disrupts perfusion particularly in the renal medulla and is considered to be a significant reason why parenchymal cells in this region (primarily S3 proximal tubule cells) are the most severely affected in these models⁷. Recent reviews have highlighted how impairment of renal microvascular function is of central importance in both initiating and predicting the severity of AKI and have called for development of new methods to better evaluate renal perfusion in susceptible patients⁶. A better appreciation for the complicated nature of endothelial dysfunction in the setting of AKI can be obtained from a number of comprehensive review articles both by us^{5,8} as well as other investigators⁹⁻¹¹. For the purposes of this article, we wish to highlight central concepts regarding impaired vascular function in the development of acute kidney injury, implications for transition from acute to chronic kidney disease, and how these may relate to the potential use of endothelial-targeted cell-based therapies.

Reductions in renal blood flow (RBF) following ischemia or hypoxia have been thought to be due, in part, to rapid vasoconstriction, perhaps secondary to impaired proximal tubular sodium reabsorption and activation of tubular glomerular feedback¹. However, if glomerular filtration rate (GFR) is reduced, sustained tubular glomerular feedback likely does not contribute to sustained vasoconstriction⁶. While activation of other vasoconstrictor pathways (e.g., sympathetic tone, angiotensin II, endothelin) or impairment of vasodilator pathways (e.g., prostacyclin, nitric oxide) have been suggested to reduce renal blood flow during AKI, the inability of vasodilators to reverse AKI suggests that these pathways may play only a modest (at best) contributory role to sustained loss of renal function⁸.

In the last 10-15 years there has been considerable attention focused on the potential role of the post-glomerular peritubular capillaries contributing toward the development of AKI. These vessels subservise reabsorption of water and solute from the interstitium back into systemic circulation and are characterized by low hydrostatic pressure. These vessels also deliver oxygen and nutrients to the renal tubular system and are in close apposition to these cells. Counter current flow in vasa recta allows for oxygen shunting in the renal medulla and development of an oxygen gradient producing a relatively hypoxic environment ⁹.

Reductions in RBF can reduce GFR and reduce tubular metabolic work, but extreme impairment may further exacerbate renal hypoxia and can activate inflammatory and cell injury pathways in nearby S3 proximal tubule or thick ascending limb ^{1,9,10}. Peritubular capillary damage may contribute further to impaired perfusion and may predict the severity and duration of AKI. In studies of transplant patients receiving cadaveric grafts, renal biopsies obtained immediately post-perfusion demonstrated evidence of both endothelial and vascular smooth muscle damage as indicated by reduced von Willebrand factor staining and actin cytoskeletal disorganization. The degree of vascular damage in the immediate post-transplant period was predictive of reduced graft function in the subsequent 7 days of recovery ¹².

Endothelial dysfunction as a contributor to AKI

Molitoris and Sutton suggested that disturbances in microvascular flow in the kidney lead to the extension phase of AKI ¹³, in which tubular damage is exacerbated by events in the endothelium which may include endothelial swelling, increased expression of adhesion molecules and associated recruitment of various leukocyte populations. Endothelial leukocyte adhesion, activated in the setting of AKI, may result from increased exocytosis of Weibel-Palade bodies ¹⁴ and an immediate enhancement of surface adhesion molecules such

as P-selectin¹⁵⁻¹⁷ and ICAM-1^{1, 18, 19 19} (Figure 1). Increased co-stimulatory factor expression is also observed on the surface of capillary endothelial cells and inhibition of B7-1 reduces monocyte infiltration in the ascending vasa recta in response to renal ischemia reperfusion²⁰.

The initiating pro-inflammatory activity in the endothelium requires further investigation. Paracrine factors such as cytokines or other danger signals from stressed epithelium represent potential links between the epithelial and vascular compartments¹⁰. Endothelial responses may also directly result from ischemia by altering endothelial cytoskeletal structure, resulting in reduced endothelial cell-cell contact or cell-adhesion complexes²¹. Mitochondrial damage following ischemia has been documented in peritubular capillaries and a recent study showed that a compound that targets mitochondrial cardiolipin attenuated endothelial mitochondrial damage and the development of inflammation following renal ischemia reperfusion²². Both ischemia and sepsis are known to induce glycocalyx shedding. The glycocalyx is considered an initial layer of endothelial barrier function, and its disruption may initiate downstream signaling cascades and increase access of leukocytes to endothelial adhesion molecules^{21, 23}. Loss of endothelial cell barrier function may also activate coagulation cascades, and potentially influence renal function by increasing interstitial edema and intra tubular pressure²³.

Leukocyte adhesion may contribute to the development of vascular rouleaux, which manifest prominently within a few hours post-ischemia in rodent models of AKI^{1, 10}. Vascular congestion and rouleaux have also been described in dogs following renal I/R²⁴ and in monkeys following LPS induced AKI²⁵. Further, Solez et al., demonstrated significant intravascular leukocyte accumulation in 63 of 66 cadaver kidneys from patients with established AKI for greater than 24 hours²⁶.

Efforts to improve renal perfusion in acute kidney injury have historically been directed toward managing fluid volume and cardiac output. While increased vasomotor tone may reduce GFR in the initial development of AKI, renal vasodilators have been shown to lack efficacy in improving renal function in established AKI^{27,28}. We recently suggested that the failure of vasodilators to alleviate established AKI is based on the inability of such therapies to resolve vascular congestion/ inflammation and that potential treatments should target established endothelial/leukocyte interaction⁸. In support of this idea, retrograde hydrodynamic delivery of saline at high pressure into the post-glomerular vasculature of rats alleviated vascular congestion, improved capillary perfusion and resulted in more rapid recovery of serum creatinine following the establishment of AKI²⁹.

Effects of renal injury on peritubular capillary rarefaction and the AKI to CKD transition

The degree to which effective endothelial repair influences chronic renal function and the long-term sequelae of AKI has also received significant attention in the last 10-15 years. Several reports indicate that peritubular capillary density is reduced permanently following AKI despite the apparent recovery of function following the initial insult. In rats and mice, there is a 30-50% reduction in capillary density following AKI, which is proposed to promote tissue hypoxia and activate pathways associated with the development of interstitial fibrosis^{5, 30, 31} and may also contribute to the sensitivity of post-ischemic rats to develop hypertension³². Sustained capillary rarefaction has emerged as a potential common feature present in nearly all models of chronic renal injury associated with interstitial fibrosis³³. Capillary rarefaction is present in human CKD of a variety of different etiologies and the loss of capillaries is strongly correlated with interstitial fibrosis³⁴. Thus it is conceivable that impaired renal microvascular perfusion may represent a potential biomarker to predict the

onset and severity of CKD. Chade and colleagues have demonstrated that CT imaging can be used to detect deficits in renal perfusion associated with vascular rarefaction using a swine model of renal artery stenosis³⁵. Similarly, MRI techniques have shown promise to identify differences in renal perfusion patterns in patients with mild CKD vs. healthy control patients³⁶. These observations suggest novel imaging approaches could be used to measure impaired renal perfusion and identify patients at risk of progression.

While hypoxia secondary to vascular dropout appears to contribute to the development of renal fibrosis following AKI, there is clearly complex interplay involving multiple cell types that culminate in the AKI to CKD transition^{1,37}. For example, in recent years, it has been suggested that pericyte detachment from damaged peritubular capillary endothelial cells trigger their activation to pro-fibrotic myofibroblasts representing the primary cell type contributing toward extracellular matrix (ECM) production³⁸.

In addition, recent evidence supports the view that persistently damaged tubular epithelium contributes to the development of fibrosis (reviewed extensively in^{37,39,40}). Interestingly, significant damage specifically in proximal tubule cells using a transgenic diphtheria toxin receptor transgenic mouse model was shown to result in persistent inflammation, activation of fibroblasts and interstitial fibrosis⁴¹. It is thought that dedifferentiated cells and/or cells which become arrested in G2/M phase of proliferation produce pro-fibrotic mediators such as TGF- β or CTGF, interact within the interstitial environment and stimulate the production of ECM^{39,40}.

The failure of epithelial cells to fully repair may represent a deficit of the tubular cell itself, however we suggest such failed recovery may also result from the lack of appropriate local perfusion secondary to capillary rarefaction. Indeed, Suzuki et al., demonstrated in a model of microembolism that heterogeneously damaged peritubular capillaries were in close apposition to areas of simplified epithelia and increased interstitial cell density⁴². Taken

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together the development of interstitial fibrosis in the AKI to CKD transition can be viewed as complex interaction of damaged vascular, epithelial and interstitial cell types, and the targeting of any of these pathways could be envisioned to promote more successful repair and improved long-term outcomes ².

Causes of capillary rarefaction following renal injury

If reduced peritubular capillary density represents a significant contributor toward progression of CKD, then an understanding of the pathophysiological loss of capillary density following an injury may help identify potential therapeutic strategies. The cause of capillary rarefaction is not firmly established, but it likely involves endothelial mesenchymal transition (EndoMT), combined with a relative lack of compensatory endothelial proliferation^{43,44} (Figure 2). These activities may result from a combination of a reduced angiogenic environment and trophic support from surrounding tubules or pericytes⁴⁵⁻⁴⁷. Goligorsky and his colleagues have suggested inhibition of eNOS could trigger EndoMT⁴⁸ and that in the setting of vascular stasis, reduced shear stress may be a trigger for EndoMT and vascular rarefaction⁴⁹. Patschan et al., suggested that EndoMT may result from endothelial cytoskeletal alterations in response to ischemia, which reduces α -tubulin expression and is associated with impaired endothelial cilia function⁴⁴. Interestingly, in animal models of AKI, EndoMT and rarefaction of capillaries is sustained despite ongoing renal tubular repair and recovery of renal function. For example, our laboratory demonstrated evidence of EndoMT for up to 7 days following I/R in rats, while Ehling et al., demonstrated the nadir of capillary density was not reached until approximately 14 days following I/R, a time point well beyond the normal re-establishment of plasma creatinine to normal values^{50,51}.

We have suggested that sustained capillary rarefaction may be attributable to the lack of resident renal endothelial progenitor activity, representing the basis for modest EC proliferation and sustained rarefaction observed following injury⁵⁰ (described further, below). Interestingly, VEGF121 treatment post I/R prevented capillary rarefaction by attenuation of EndoMT but did not influence kidney endothelial proliferation⁴³. Therefore, given the sustained duration of EndoMT process contributing to capillary loss following recovery from acute injury, a critical therapeutic window may be available to treat patients recovering from AKI to improve capillary survival and long term renal function.

Taken together, the issues highlighted above indicate a need for therapeutic approaches to address endothelial function to treat both the acute as well as the chronic vascular damage resulting from AKI. The remainder of this article will focus primarily on the potential role of stem and progenitor cells targeting endothelial function and the potential therapeutic applications of these cells in the setting of acute kidney injury.

Defining cells types with potential for endothelial repair potential

Numerous studies invoking a role for endothelial progenitor cells (EPC) in renal vascular repair in both acute and chronic kidney disease have been published. However, the field has been hampered by inconsistent definitions and different methodologies used to isolate or identify various cell types classified as EPCs. Because of the variety of different cell types used in these studies, we endorse previous suggestions to abandon the term “endothelial progenitor cell” in favor of more precise terminology to allow for better interpretation and comparison of the results from different studies⁵². In general, cells with particular relevance to angiogenic repair can be classified into two broad categories as either hematopoietic or endothelial in origin and these distinctions are described in greater detail below.

Historically, most studies have referred to EPCs as bone marrow-derived cells, which express cell surface markers common to endothelial cells and may mediate pro-angiogenic or vascular repair activity⁵³ (Figure 3). Several populations of cells have been described and characterized by the nature of their biological source, isolation procedure and the presence of various cell surface markers which provide insight into their origin. Hematopoietic stem cells (HSC) give rise to multiple lineages of myeloid and lymphoid cells, many of which display pro-angiogenic activity⁵⁴⁻⁵⁷. Therefore different selection criteria may lead to isolation of upstream progenitors with the potential to differentiate into more terminally differentiated cells which may modulate vascular injury. While HSC differentiate into a variety of different myeloid and lymphoid populations, advances in confocal imaging to improve spatial localization of transplanted cells⁵⁸ and the use of lineage specific reporter transgenic murine models⁵⁹⁻⁶¹ or analysis of epigenetic regulation in endothelial cells⁶² has eliminated any evidence that these cells are capable of differentiating into endothelial cells. Nevertheless, their involvement in processes such as vascular remodeling⁶³⁻⁶⁵ make them particularly relevant in understanding potential effects in the setting of acute kidney injury.

Early outgrowth/ CFU Hill cells: As originally described, EPCs are isolated from either low density mononuclear cells or CD34+ or CD133+ enriched cells, which are cultured for several days on fibronectin coated plates. The cells express markers of endothelial cells such as CD31, CD105, CD144, CD146, vWF and KDR⁶⁶. These cells have subsequently been referred to as “early outgrowth” cells or colony forming unit Hill (CFU-Hill) cells⁶⁷⁻⁷⁰. The cells have generally been shown to be effective at promoting vascular repair via paracrine secretion of proangiogenic molecules in preclinical models of vascular repair where they typically improve or hasten remodeling⁷⁰. These cells are now all recognized to be various stages of hematopoietic cells with proangiogenic potential⁷⁰⁻⁷².

Hematopoietic pro-angiogenic cells: Putative endothelial progenitor activity from cells isolated based on their expression of surface markers of CD34, CD133 and the VEGFR2 (KDR) has been reported⁶⁸. Many studies report that these cells augment angiogenic repair in vascular injury models^{67, 73, 74} leading to the suggestion that these cells contain endothelial progenitor activity. However, careful examination has demonstrated that CD34/CD133/VEFR2 cells possess hematopoietic colony forming activity^{75, 76}. While neither the CD34/CD133/VEFR2 expressing cells or CFU-Hill cells have been shown to become bone fide endothelial cells by incorporating into the endothelial layer of a repaired vessels, the ability of the CD34/CD133/VEFR2 expressing cells to promote vascular repair, and the ability to isolate these circulating cells from blood has suggested that these may serve as a potential biomarker for overall cardiovascular risk^{77, 78}.

Circulating Angiogenic Cells: This term has been applied to a variety of hematopoietic stem and progenitor cells that display the capacity to secrete proangiogenic factors that participate via paracrine mechanisms to promote vascular repair and regeneration. However, this term may more broadly include non-hematopoietic cells present in the circulation such as rare endothelial colony forming cells (cECFC) or even mesenchymal cells. In essence any cell that can circulate in the bloodstream that produces proangiogenic molecules may be generally referred to as a circulating angiogenic cell; thus, the term lacks specificity and as such fails to provide the reader with clarity about the cell type being examined^{52, 79}.

Myeloid angiogenic cells: This term represents a population of cells derived from peripheral blood derived cultures which express markers of myeloid progenitor cells, monocytes, or macrophages, but do not express markers of hematopoietic stem and progenitor cells and do not proliferate significantly ex vivo⁸⁰⁻⁸². Of interest, these cells do

express potent angiogenic growth factors and participate in vascular remodeling and repair⁸³.

This term is a preferred choice for describing the non-ECFC types of angiogenic cells that participate in paracrine support of vascular repair according to a recent consensus statement.⁴⁰

Endothelial colony forming cells. This term identifies rare circulating viable endothelial cells that give rise to colonies of endothelial cells when peripheral blood cells are cultured *in vitro*^{84, 85}. While most circulating endothelial cells are undergoing apoptosis or necrosis in the bloodstream⁸⁶, very rare viable cells possess this endothelial colony forming cell (ECFC) ability⁸⁵. These cells have been isolated from human, baboon, rhesus monkey, cow, sheep, dogs, pigs, and rabbit peripheral blood (reviewed in⁸⁷). Human ECFC have been delivered in numerous animal models of human disease and display postnatal vasculogenic properties by making human blood vessels in all the models in which the ECFC are delivered directly into ischemic or injured tissue^{88, 89}. When ECFC are delivered intravascularly into injured animals, little engraftment has been observed and the protective effects to enhance vascular recovery has been demonstrated to reside in secreted molecules released by the ECFC or contained in their exosomes^{90, 91}¹⁹.

A variety of different cell types influence endothelial responses to renal injury

Based on the description above, we sought to classify studies based on whether the cells of interest could be identified as either pro-angiogenic cells, endothelial colony forming cells (ECFC) or endothelial cells (EC). The term pro-angiogenic cells is meant to convey any cell of hematopoietic origin with potential vasculogenic activity and would therefore encompass cells such as early outgrowth “endothelial” cells (eOEC) or myeloid angiogenic cells. In some cases, we were unable to be sure which of these categories was isolated by the

authors of the numerous papers that have delivered “EPC” into murine models of AKI and therefore categorized the cell type as indeterminate (Table 1). It is of interest that exogenous delivery of both pro-angiogenic cells and ECFC possess protective activity in models of AKI.

Pro-angiogenic cells. Interest in bone marrow-derived pro-angiogenic cells arises from studies demonstrating the localization of cells expressing both hematopoietic and endothelial markers at sites of vascular injury as well as studies indicating that exogenously administered pro-angiogenic cells influence outcomes in various models of vascular injury. Early outgrowth EPC or blood derived CD34+ cells home to sites of injury, improve perfusion and stimulate angiogenesis in models of ischemia of the myocardium or hindlimb^{80,92}. The basis for the pro-angiogenic environment created by BM-derived cells is thought to be the production of factors such as VEGF, HGF or G-CSF^{53,92-94}. Pro-angiogenic cells have been shown to limit neointima formation and improve arterial reactivity following aortic balloon injury⁹³. Since these cells do not differentiate into endothelial cells, the current view is that the pro-angiogenic environment created by these cells activates proliferation and migration of tissue resident HPP-ECFC (high proliferative potential-endothelial colony forming cells) to facilitate recovery from injury (Figure 4).

In kidney, selective injury to the renal vascular endothelium via infusion of concanavalin A into the renal artery was shown to induce homing of bone marrow derived CD34+/Flk1+ positive cells, which were referred to as EPCs. Mobilization of these cells was evident within 3 hours in the blood and infiltration into the kidneys established within 3-5 days⁹⁵. Mediators of this homing activity have been suggested to include secretion of cytokines such as IL-8, G-CSF or GM-CSF. Injury induced up-regulation of the chemokine SDF-1 was shown stimulate homing of pro-angiogenic cells in CXCR4 dependent fashion

following I/R injury in mice ⁹⁶, while others have suggested that the up-regulation of adhesion molecules on the surface of damaged endothelial cells may promote homing of these cells to injured kidney ⁹⁷. Finally, uric acid, which is produced abundantly by ischemic kidney and is elevated in pigs with renal artery stenosis ⁹⁸, has also been suggested as a potential homing signal, since administration of a uricase inhibitor blunted the mobilization of cells into mouse kidney following I/R ⁹⁹.

Bone marrow derived pro-angiogenic cells appear to influence renal vascular function in the setting of AKI (Figure 5). Patschan and colleagues demonstrated increased levels of “EPC”-activity, defined as c-Kit+/Tie-2+ cells in a mouse model of ischemic preconditioning. When these cells were isolated from preconditioned kidneys, they conveyed protection in recipient mice subjected to I/R injury ¹⁰⁰. Other studies using exogenous pro-angiogenic cells (derived from isolated and cultured mouse mononuclear cells), demonstrated similar effects on protection from renal injury, but did not directly assess vascular function ^{101, 102}. Recently, administration of these cells was shown to attenuate peritubular capillary rarefaction post I/R, in part by inhibition of EndoMT ⁴⁴.

Other preparations of hematopoietic stem cells with putative pro-angiogenic activity have led to conflicting results on the outcome of renal injury. For example, Burger et al., isolated CD133+ cells from human cord blood, of which up to 83 percent of the cells were CD34+, 98% were CD45+, while only 26% were KDR positive. Administration of these cells to NOD SCID mice following renal I/R worsened changes in serum creatinine, tubular damage and promoted inflammation, while the cells did not home to the kidney ¹⁰³. These studies suggest that isolation based on CD133+ alone includes diverse hematopoietic progenitor cells that increases the inflammatory response to AKI rather than promoting protection.

In contrast, Li et al., studied HSPC from adult volunteers pretreated with G-CSF to promote HSC mobilization. Mobilized CD34+ cells enriched from peripheral blood mononuclear cells by magnetic separation were primarily CD45+ and only a small percentage expressed CD14, CD133, CD146, CD31 or KDR. However transplanted cells homed to kidney 24 hours following renal ischemia reperfusion injury, and improved renal function and survival in NOD SCID mice. Many cells were localized in the perivascular area between 2-3 days post-injury and expressed markers of endothelial cells such as human hCD31 or KDR, however these exogenous cells were essentially absent by 7 days post-injury¹⁰⁴. Interestingly, in human transplant patients, a transient elevation in recipient CD34+ cells were observed frequently along the peritubular capillary lining within 2 weeks of ischemia, while no evidence of recipient cells were observed in the vasculature after 35 or 73 days¹⁰⁵. Taken together, these data support the view that transient homing of bone marrow derived pro-angiogenic cells migrate to areas of vascular injury and may stimulate remodeling, but these cells do not stably integrate into the vasculature, suggesting they do not act as *de facto* progenitor cells.

Despite this, these cells may stimulate endothelial cell proliferation and migration, and have been shown to induce angiogenic branch formation in vitro and in vivo^{53, 92-94}. These cells are thought to subserve an important homeostatic function. Goligorsky and colleagues have articulated that the concept of “EPC incompetence”, based on studies demonstrating that the number or activity of bone marrow derived pro-angiogenic cells is impaired in patients with increased cardiovascular risk factors. Vascular impairment in these patients can be thought of as a result of reduced activity or mobilization of these cells to maintain vascular homeostasis, a viewpoint consistent with the increased susceptibility of patients with CKD to develop AKI¹⁰⁶.

Endothelial colony forming cells

As described above, endothelial colony forming cells (ECFC), often referred to as “late outgrown endothelial cells” have been isolated following culture of blood cells on collagen following removal of non-adherent monocytes and subsequent expansion⁵³. ECFC express classic markers of endothelial cells including CD31 and VEGFR2, as well as other markers. In contrast to hematopoietic pro-angiogenic cells, ECFCs do not express markers such as CD45 and are capable of forming and stably integrating into functional vessels in vivo^{53, 55, 107}.

ECFCs can be classified based on their proliferative potential in single cell colony forming assays, in which high proliferative potential (HPP) ECFC will form large colonies (>10,000), while low proliferative potential (LPP) ECFC form small colonies (<2000).

ECFCs can be isolated and expanded from blood of humans and other large species, but cannot be isolated from blood of rodents⁵⁵. However, ECFC can also be isolated from tissues of a variety species, including rodents. This observation has led to the hypothesis that a cooperative interaction between infiltrating pro-angiogenic cells of hematopoietic origin work to provide a trophic environment to stimulate local ECFC progenitor activity to stimulate vascular repair¹⁰⁸ (Figure 4). Interestingly, our data in rats failed to demonstrate evidence of HPP-ECFC populations in kidney; rather we found only evidence of cells capable of forming small colonies, i.e., low proliferative potential ECFC⁵⁰. These observations combined with the lack of BrdU+ capillary endothelial cells following renal I/R⁴³ suggest that a low degree of endogenous ECFC activity may contribute to impaired recovery and maintenance of vascular rarefaction following AKI (Figure 2).

Because ECFC represent true endothelial progenitors, there is considerable interest in exploiting these cells for potential therapeutic effects. Human cord blood represents one of the richest sources of HPP-ECFC⁸⁵ and recent studies also demonstrate that iPS cells can be

differentiated into highly active HPP-ECFC⁸⁸. To date, the potential therapeutic benefit of ECFC has been less well studied in preclinical models of vascular impairment than hematopoietic pro-angiogenic cells. Nevertheless, ECFCs stimulate neovascularization in a hindlimb ischemia model¹⁰⁹ and attenuate the development of pulmonary hypertension in a rat model of arrested alveolar development⁹⁰

ECFC appear to effectively ameliorate the severity of injury in models of AKI (Figure 5), an observation gleaned initially from studies in which the influence of HUVEC administration was assessed in a model of I/R. HUVEC rapidly expand in culture and contain a significant population of HPP-ECFC¹⁰⁸. In these studies, systemic infusion of HUVEC in athymic rats following I/R injury significantly improved capillary flow rates as observed by video microscopy^{110,111}. HUVEC infusion also resulted in a significant protection against the loss of renal function (e.g., by serum creatinine) and tubular injury. Surrogate non-endothelial cells had no effect on I/R induced damage, but when cells overexpressed eNOS, there was an improvement in renal blood flow leading to the suggestion endothelial supplementation influenced AKI via the nitric oxide pathway^{102,103}.

Recent results from Burger et al., support the suggestion that ECFC have renal protective properties. Using human cord-blood derived HPP-ECFC injected immediately following ischemia reperfusion, AKI was attenuated in SCID mice as assessed by creatinine, tubular necrosis, macrophage infiltration and oxidative stress¹¹². In contrast to results obtained with bone marrow derived pro-angiogenic cells, ECFCs showed very little evidence of homing into the kidney. Similarly, our group recently demonstrated that rat pulmonary microvascular endothelial cells (PMVEC), which have a high level of HPP-ECFC, failed to home to the kidney but protected Sprague Dawley rats from I/R induced AKI¹⁹. In contrast, studies by

Pang et al., demonstrated that a small number of EC/ECFC from isolated from human renal artery migrate into the renal peritubular region of SCID mice following severe I/R injury ¹¹³, suggesting that the fate of transplanted EC/ECFC following kidney injury is not clearly understood.

It is of interest that low proliferative potential ECFC (from pulmonary artery) had no protective effect on ischemia reperfusion injury ¹⁹, indicating that the protective effects are a function of the proliferative capacity of ECFC. Interestingly, Patschan et al., found only minimal protection from AKI by late outgrowth cells obtained from mouse peripheral blood and spleen ¹¹⁴. These cells expressed markers of EC such as VE-cadherin, but since ECFC do not circulate in mice ¹¹⁴, these cells likely had low proliferative potential relative to other studies demonstrating protective effects of ECFC.

The lack of homing of ECFC suggests that secreted factors may promote resistance to AKI. Exosomes derived from ECFC conditioned media improve renal function post ischemia and prevent endothelial injury to hypoxia *in vitro* ¹¹². ECFC derived exosomes contain a high level of microRNA486-5p which may mediate these protective effects by decreasing endothelial PTEN (Phosphatase and tensin homolog) and increasing Akt ⁹¹. Other investigators have demonstrated microvesicles (MV) from conditioned media of human blood derived EC protected Wistar rats from AKI, while micro-vesicles derived from fibroblasts did not. Treatment of MV with Dicer or antimir 126 or 296 blocked the protective effect of microvesicles ¹¹⁵. The authors proposed a so-called “horizontal transfer” of information of miRNA from micro-vesicles into the endothelium as the basis by which protection is mediated ^{115, 116}. We recently demonstrated that rat PMVEC or conditioned media of human ECFC protected against early alterations in renal hemodynamics following ischemia reperfusion injury, by preventing the loss of renal medullary blood flow. Human ECFC

conditioned media also prevented the immediate up-regulation of endothelial ICAM-1 and the rapid infiltration of T-lymphocytes into the kidney within hours of ischemia reperfusion¹⁹.

Despite protective effects of both bone marrow derived pro-angiogenic cells and ECFC in acute kidney injury, whether these strategies can be used to effectively reverse the rarefaction of vessels associated with AKI and prevent progressive CKD has not been explored. The paradigm of cooperative interaction¹⁰⁸ (and Figure 4) highlights the role of local ECFC in the vascular repair response which could be stimulated by pro-angiogenic cell-treatment.

Future Directions

We hypothesize that a limited endogenous kidney ECFC proliferative potential contributes to impaired vascular repair and sustained rarefaction following injury. Whether cell-based therapies may have a future in treating patients with AKI is not yet clear. While preclinical data using ECFC, pro-angiogenic cells or their conditioned media indicate the potential to preserve vascular function in AKI, translating these observations to the clinic represents a significant hurdle. We propose cell-based therapies in AKI, or any therapeutic study in AKI, should not be restricted to acute hospital outcomes but incorporate the potential development of subsequent CKD following discharge of AKI survivors, which may take months or years to manifest. We envision that agents with little obvious acute protective effect in the recovery period may provide long-term benefit and that such goals should be considered in clinical study design.

From a revascularization point of view, the inability of ECFC to home to the kidney following I/R hampers efforts to facilitate vascular remodeling and is a hurdle that must be overcome. By extension, supplementing with high proliferative ECFC may result in improved vascularization, if retention and integration could be achieved. Future studies

should investigate how improved methods of administration of cells could help overcome barriers toward homing of ECFC and facilitate repair. It is possible that a combination of bone marrow derived pro-angiogenic cells with ECFC may help to revascularize the kidney. In support of this hypothesis, co-culture of these two cell types promoted cooperative formation of capillary-like structures in Matrigel plugs, while co-adoptive transfer of both cell types in hind limb ischemia promotes greater *in vivo* neo-angiogenesis when compared to a single cell transfers alone ¹¹⁷. It is of interest that Chade et al., have demonstrated that administration of endothelial progenitors described as a mix of “early” and “late” outgrowth cells directly into the renal artery of swine with renal artery stenosis improved in renal vascular structure, renal function and an attenuated renal fibrosis ¹¹⁸.

New approaches such as the use of stromal vascular fraction (SVF), which contain both ECFC like cells and pro-angiogenic trophic cells could be used as SVF has been shown in animal models to attenuate the development of AKI and progressive CKD following I/R ¹¹⁹. Similarly, we demonstrated that human adipose derived stromal cells could prevent the loss of renal peritubular capillaries and limit renal fibrosis up to 1 week after recovery from renal ischemia reperfusion in rats ¹²⁰. Therefore, a combination of different cells with cooperative activities could be envisioned to improve vascular repair or prevent capillary loss following kidney injury as a means to enable better long term renal function.

Finally, it will be important to more fully understand the biological basis for low level of ECFC proliferative potential in kidney. At the current time, these progenitor cells can only be effectively studied and identified *ex vivo* using clonal analysis. No unique set of markers has yet been identified for HPP ECFC, although efforts are clearly underway to identify key genes regulating ECFC activity ⁸⁸. If proper markers can be identified, it may enable efforts

to study the nature of ECFC activity in the kidney, the pathways that subserve the high proliferative potential, and even perhaps to devise strategies to influence progenitor activity of the renal EC themselves.

Acknowledgements:

Support for studies by the authors reviewed in this paper are summarized as follows: DPB was supported by NIH grant DK-063114 and Fortune Fry Fund and Bridge Funding from the Indiana University Research Foundation and pilot funding from the George M O'Brien Center P30-DK79312 (Molitoris, program director). Support for JAC was by NIH T32 HL079995-10 (K. March, PI) and an NIH/CATS postdoctoral fellowship TL1R001107 (A. Shekar, PI). MCY was partially supported by funds from the National Institutes of Health grant DK106846 and National Center for Advancing Translational Sciences grant TR000006 and the Riley Children's Hospital Foundation.

Conflict of Interest: The authors report no conflict of interest.

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Table 1.

Author, reference, year	Cell Type	Biological Source	Model/Biological Effect
Patschan <i>et al.</i> ¹⁰⁰ 2006	Pro-angiogenic	Murine spleen and kidney	Unilateral I/R in FVB/NJ and Tie-2-GFP mice Reduced plasma creatinine by ~50% post I/R No evidence of homing
Patschan <i>et al.</i> ¹⁰¹ 2010	Pro-angiogenic	Murine peripheral blood, spleen and bone marrow	Unilateral I/R in male C57BL/6N mice Reduced plasma creatinine by ~40-75% post-I/R Reduced plasma creatinine by ~90% post-I/R with Epac-1 Ac pretreatment Evidence of minimal homing
Patschan <i>et al.</i> ¹²¹ 2012 Patschan <i>et al.</i> ¹²² 2013 Patschan <i>et al.</i> ¹²³ 2013		Murine peripheral blood and spleen	Similar models as above; pretreatment of cells with melatonin, Angiotensin-1 and BMP-5 enhances protective response, with minimal homing
Patschan <i>et al.</i> ¹²⁴ 2015 Patschan <i>et al.</i> ⁴⁴ 2016	Pro-angiogenic	Murine peripheral blood and spleen	Bilateral I/R in male C57BL/6N mice, reduced plasma creatinine, increased endothelial autophagy and decreased EndoMT and attenuated capillary rarefaction
Burger <i>et al.</i> ¹¹² 2015	ECFC & ECFC exosomes	Human umbilical cord	Bilateral I/R in male NOD-SCID mice Reduced plasma creatinine by ~40% post-I/R Improved tubular morphology No evidence of homing
Collett <i>et al.</i> ¹⁹ 2017	ECFC	Rat PMVEC and human ECFC-conditioned	Bilateral I/R in male Sprague Dawley rats Reduced plasma creatinine by ~50% post-I/R

		media	Improved tubular morphology Prevented loss of medullary blood flow No evidence of homing
Viñas <i>et al.</i> ⁹¹ 2016	ECFC	Human umbilical cord blood ECFC derived exosomes	Bilateral I/R in male FVB mice Reduced plasma creatinine by ~90% post-I/R Improved tubular morphology
Brodsky <i>et al.</i> ¹¹¹ 2001	EC	Human umbilical cord	Unilateral I/R in male Athymic rats Reduced plasma creatinine by ~50% post-I/R Maintained microvascular perfusion Evidence of minimal homing
Cantaluppi <i>et al.</i> ¹¹⁵ 2012	ECFC derived microvesicles	Human peripheral blood	Unilateral I/R in male Wistar rats Reduced plasma creatinine by ~80% post-I/R Improved tubular morphology Prevented capillary rarefaction
Liang <i>et al.</i> ¹²⁵ 2015	EC	Human Umbilical cords (Wharton's Jelly)	Unilateral I/R in male C57BL/6 mice Reduced plasma creatinine by ~70% post-I/R Improved tubular morphology Potential prevention of capillary rarefaction Evidence of minimal homing
Pang <i>et al.</i> ¹¹³ 2017	EC or ECFC	Human renal artery	Bilateral I/R and nephron mass reduction in NOD-SCID mice. Reduced plasma creatinine by ~50% post-I/R Prevented capillary rarefaction Minimal homing near peritubular capillaries up to 10 days
Patschan <i>et al.</i> ¹¹⁴ 2017	Indeterminate	Murine peripheral blood and spleen	Bilateral I/R in male C57BL/6 mice Reduced plasma creatinine by ~10% at 1 week, did not prevent capillary rarefaction
Zullo <i>et al.</i> ¹²⁶ 2015	Indeterminate	Murine embryonic EPC line cultured with MSCs	Improved renal function, medullary RBF and increased M1 to M2 macrophage polarization in LPS induced endoxemia in C57BL6 mice.

ECFC-endothelial colony forming cells; EC-endothelial cells

Figure Legends

Figure 1. Alterations in endothelial function contribute to the extension phase of acute kidney injury. On the left, a peritubular capillary is shown in close apposition to tubular epithelium in a normal kidney. B) In response to injury, endothelial swelling narrows capillary space. Increased adhesion molecule expression facilitates leukocyte attachment, contributing to erythrocyte rouleaux formation and disrupting normal blood flow. Reduction in flow contributes to reduced shear stress and inhibition of NO formation, a potential trigger for endothelial mesenchymal transition (EndoMT). Additional potential contributors toward EndoMT include a reduction in trophic support from damaged tubules or injury activated pericytes. Disruption of blood flow exacerbates tissue hypoxia and further compromises epithelial injury and a reduction in renal

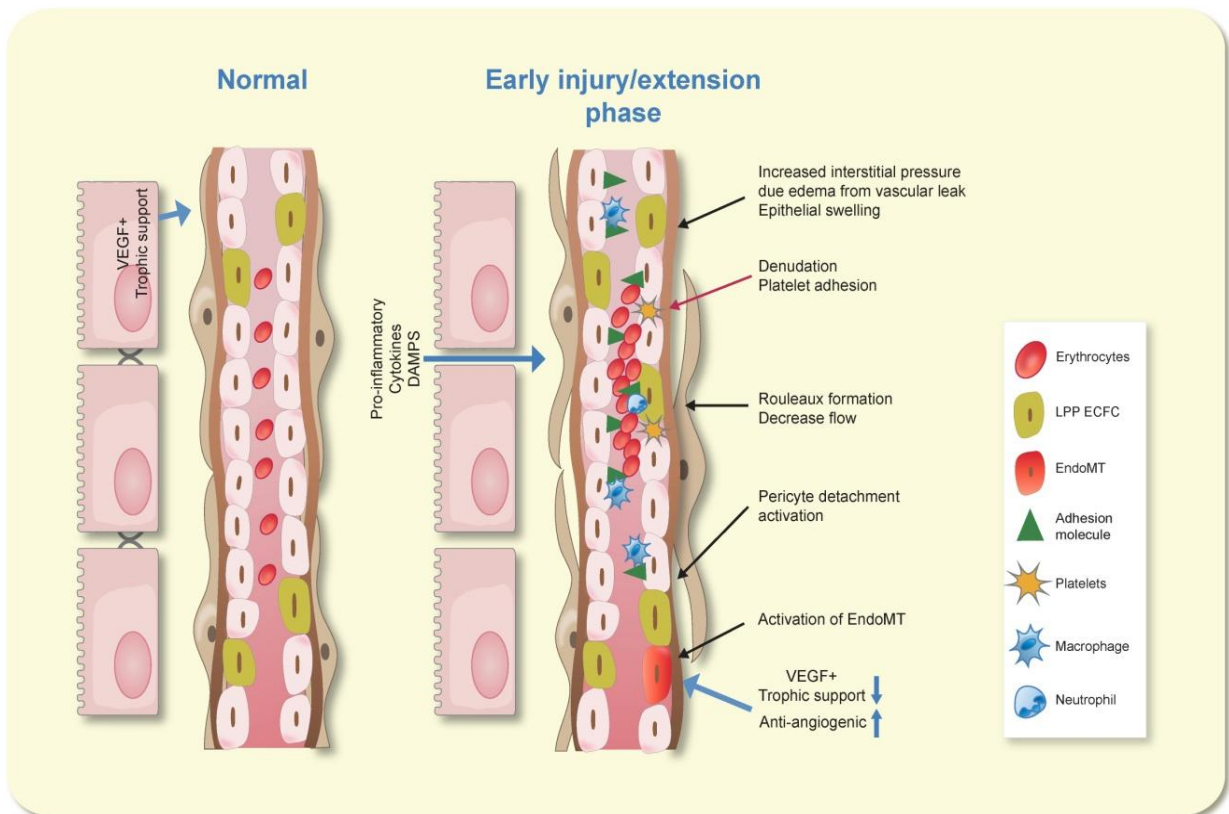
Figure 2. Failed vascular recovery leads to peritubular capillary rarefaction following AKI. Concurrent with resolution of GFR and tubular repair recovery of the capillary endothelium is inefficient due to a combination of EndoMT and low endothelial proliferation. Infiltration of pro-angiogenic hematopoietic cells provide pro-angiogenic stimulation but renal endothelium is unresponsive due to the lack of HPP-ECFC activity intrinsic in kidney. Expansion of fibroblasts or myofibroblasts, which derive from either pericyte activation or EndoMT, may occlude blood vessels leading to a rarefied capillary bed.

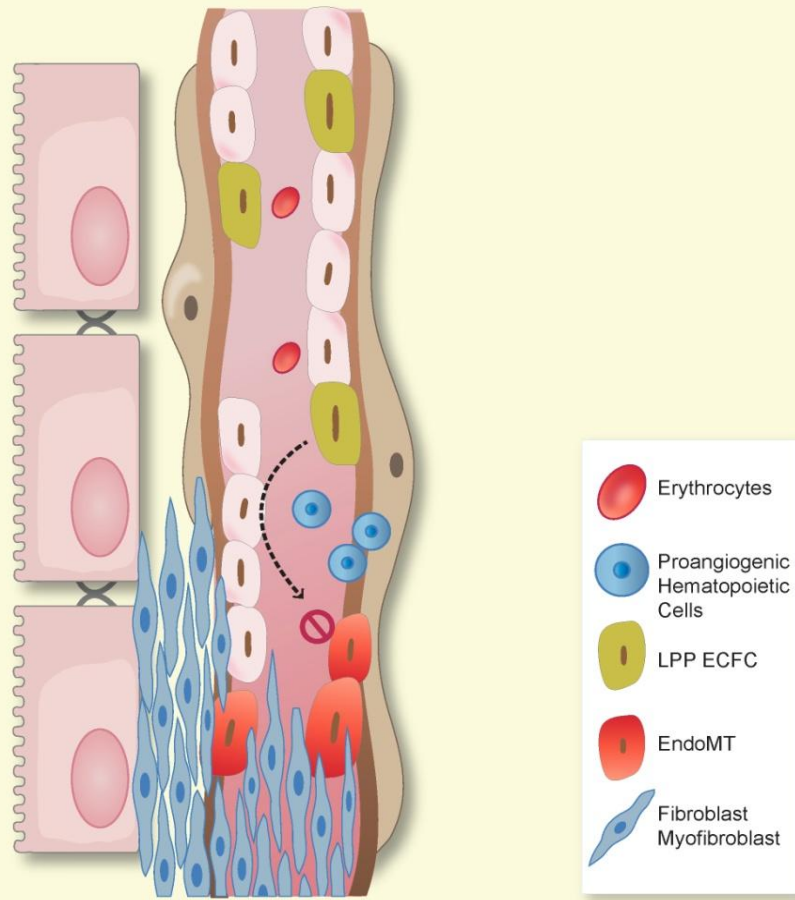
Figure 3. Human “endothelial progenitor cells” (EPC) have been identified using monoclonal antibodies or specific ligands to detect numerous cell surface antigens. Three key antigens CD34, AC133, and KDR are the most frequently utilized as markers for human EPC. No unique antigen has been reported that can discriminate the human EPC from other cell lineages (many of the above antigens are present on blood cells).

Figure 4. Proangiogenic hematopoietic cells (PHC) do not become endothelial cells but do stimulate resident endothelial colony forming cells (ECFC) to repair the intima. Moving from the left to the right on the image, one can visualize that any denudation injury that causes loss or turnover of the resident endothelial cells results in an area of exposed subendothelial basement membrane. Circulating platelets would be readily recruited to the exposed basement membrane and would release chemokines and growth factors to recruit circulating PHC, ranging from bone marrow progenitor cells to mature circulating monocytes and neutrophils, to assist in adhering to the basement membrane. The recruited PHC secrete a host of growth factors, chemokines, and proteolytic enzymes to stimulate the proliferation and migration of resident ECFC into the site of the injury to reconstitute the endothelial barrier and promote normal homeostatic functions through the injured vessel segment. The PHC merely migrate into the tissue where they can differentiate into mature tissue resident cells, re-enter the circulation, or undergo senescence and are cleared by macrophages in the tissues.

Figure 5. Both HPP-ECFC and pro-angiogenic hematopoietic cells can mitigate acute kidney injury. Both cell types have been shown to improve renal function and renal perfusion when administered prior to the establishment of renal injury. Possible mechanisms include a direct inhibition of adhesion molecule expression proposed to maintain perfusion in the early injury phase (See Figure 1). Maintenance of vascular structure likely is based on prevention of endothelial loss for which EndoMT represents a primary mechanism.

Protection is likely mediated by released factors such as pro-angiogenic factors or exosomal transfer of microRNA to protect endothelial injury. Whether administered ECFC could repopulate capillary endothelium is currently subject to debate (likely depends on mode of administration). We propose that co-operative activity of both HPP-ECFC and pro-angiogenic hematopoietic cells could potentially lead to successful engraftment in the acutely injured kidney.





Markers previously used to identify EPC

